A NEW APPROACH TO THE PROBLEM OF AGGREGATION IN THE CELLULAR SLIME MOLDS[1]

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The prime reason for initiating this project was a conviction that we will never begin to understand the mechanism of aggregation in the cellular slime molds and the subsequent developmental stages unless we know the chemical identity of acrasin, the chemotactic agent responsible for the attracting of the amobae to central collection points. With this object in mind a new assay was sought for acrasin which would be serviceable for a chemical analysis. As will be evident from what follows, a practical bio-assay has been developed, but we cannot be certain until the substance or substances we are testing for are completely isolated and known, whether or not we are without question testing for acrasin and not some associated substance. Either result would be a significant advance in our knowledge of slime mold development, but by the very nature of this dilemma, this paper is a progress report rather than the final analysis of a complex problem.

The details shall be presented shortly, but briefly the matter was approached by preparing stable solutions of acrasin by previously known methods primarily devised by Shaffer (1956a, 1956b). It was noticed in the presence of these solutions the amoebae moved more rapidly and furthermore this could be turned into a quantitative test, for the speed of the amoebae is proportional to the concentration of the extracted preparation. The question arises as to whether the preparation contains both an orientation factor (acrasin) and a separate rate-increasing factor, or whether acrasin does both. Despite preliminary chemical purification, we have been unable to separate the two components. Should acrasin have both of these biological effects (a matter which can only be settled when the chemical analysis now in progress is finished) then, as we shall see presently, our traditional assumptions as to how aggregation is initiated will have to be radically altered.

Before presenting this work, a short survey of previous attempts to identify acrasin should be given. The first assay was devised by Shaffer (1953, 1956b) which consisted of sandwiching amoebae, which are in the process of aggregating, between glass and a small block of agar, and adding the test solution to the meniscus. If it is active the amoebae will orient and move towards the meniscus. By this test Shaffer was able to prove conclusively that acrasin was a substance and that cell-free solutions could attract amoebae. Furthermore he showed that it was dialyzable (confirming the previous evidence of Runyon, 1942), that it was heat-

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stable, and that it was destroyed, presumably enzymatically, in the presence of live slime mold cells (which was confirmed by Sussman, Lee and Kerr, 1956).

Partly because the test is difficult to perform, and partly because it is not a quantitative assay, its use as a tool for chemical analysis is limited. Despite these shortcomings the test has been used in a number of attempts to identify acrasin: Sussman, Lee and Kerr (1956) reported two fractions which needed to be mixed in specific proportions to obtain activity; Sussman, Sussman and Fu (1958) subsequently added a third factor to the complex which also showed some activity when used alone. Wright and Anderson (1958) gave evidence that acrasin was a steroid, and Heftmann, Wright and Liddell (1959, 1960) found stigmastenol to be present in slime molds. However, doubts still remain concerning the chemical nature of acrasin and these were reinforced by the work of Hostak and Raper (1960) who showed, using another technique of Shaffer, that various alkaloids had some sort of stimulating effect on the aggregation of *Acytostelium*.

Recently Francis (1965) has devised a most interesting new quantitative assay involving the flowing of test solutions over cells aggregating under a layer of gently moving standard salt solution. The full potentialities of this test have only begun to be realized and Francis has already made some worthwhile observations on the mechanism of aggregation. The only difficulty with the assay is that it is technically delicate and demanding, a drawback it shares with the Shaffer test.

The Assay

The object of the assay is to record the speed of the amoebae bathed in the test solution. Originally this was done by following individual amoebae with a compound microscope and making camera lucida drawings at regular intervals. While this is perfectly satisfactory it was found tedious and impossible for one person to test more than one solution at a time.

To circumvent these difficulties a standard procedure was devised so that eight or ten solutions could be tested simultaneously. The method takes advantage of the fact noted by Samuel (1961) that the amoebae tend to repel one another. Therefore if they are placed in contact with the test substance in a concentrated group they will spread, the individual amoebae going in fairly straight lines away from the dense center. If their progress is measured at two instants in time, the average rate of outward movement may be easily computed.

The precise method is as follows: 1 ml. of the test solution is carefully mixed with 1 ml. of hot 4% Bacto agar (on a slide-warming plate kept at 60° C.) in a small plastic petri dish (50 × 12 mm.—Falcon No. 1006). After the agar has solidified the covers are removed from the dishes for 10 minutes to allow excess moisture on the surface to evaporate. The amount of surface moisture has a great effect on the rate of movement, as Samuel (1961) showed, and every effort to keep standard moisture conditions is desirable. Since with each set of experiments one petri dish is run as a plain agar control, it is possible to some extent to take into account these fluctuations in the humidity of the environment.

The test amoebae may be prepared by any method which produces a suspension of centrifuge-washed cells. All the liquid culture methods are entirely satisfactory. We used the method described previously (Bonner, 1947) in which the amoebae

are grown with the bacterium on a buffered 1% peptone and a 1% dextrose agar over which 2 or 3 ml. of sterile distilled water had been poured. Two of such plates are incubated at 22° C. for 48 hours and then washed free by two two-minute centrifugations at low gravitational force. The final amoebae are in a dense suspension in standard salt solution (Bonner, 1947).

These amoebae are allowed to settle on small squares of uncoated cellophane, roughly 16 mm². (Visking dialyzing membrane), which are placed on the bottom of a petri dish filled with standard salt solution. After 20 to 30 minutes settling time the squares are removed with forceps and each dried by placing it on a piece of filter paper and then taking a corner of another piece of filter paper and touching the square in the center to remove any remaining water. The square is then placed on one of the test petri dishes, amoeba side up, three such squares being placed in each dish.

After about one hour it is possible to see that the amoebae have crossed the edge of the cellophane onto the agar. With a pair of dividers 6 grooves 2 mm. in width are scratched on the bottom of the plastic dish perpendicular to 6 sides of the cellophane squares. Then with the use of an ocular micrometer in a dissecting microscope the distances between the edge of the cellophane and the four cells farthest out are measured and averaged for each of the 6 grooves. The same procedure is repeated after two or four hours and in this way it is possible to have an index of the rate of outward movements of cells which can be expressed in terms of mm./hr. It should be pointed out that one of the advantages of the cellophane square is that it provides sharp starting lines for the spreading amoebae (Fig. 1A).

In order to give some idea of the consistency of this test, four different sets of data have been chosen at random from our notebook and presented in Table I. For each there is one control and one experimental. As a convenient rule of thumb it was considered doubtful that the difference between experiments and controls was significant if their ranges overlapped at all. It is not obvious from the small amount of data in the table what might be the advantages of the four- as compared to the two-hour reading. If a comparison is made of the size of the ranges of one-, two,- and four-hour measured intervals it was found that the mean range was 0.17 for one-hour intervals, 0.09 for two-hour intervals and 0.07 for four-hour intervals. In other words the four-hour interval may be more precise than the two-hour, but if the difference between the experimental and control is marked, this extra precision is hardly necessary.

One of the first questions that arose was to what extent the result was affected by the age of the amoebae, for unless some accurate method is used such as the Gerisch (1959, 1960) liquid culture method, the physiological age of the amoebae we use must vary considerably. In 5 experiments 24-hour amoebae were compared with 48-hour amoebae and there was no significant difference in the result. In some cases the amoebae were clearly very advanced and began aggregation immediately, yet those cells that escaped from the cellophane squares did so at the same rate as younger cells. All the tests so far indicate that test cells of any age give similar results on the same test agar.

Another variable examined was cell density but again the result was identical, regardless of the density of the cells on the cellophane block. If the cells were too

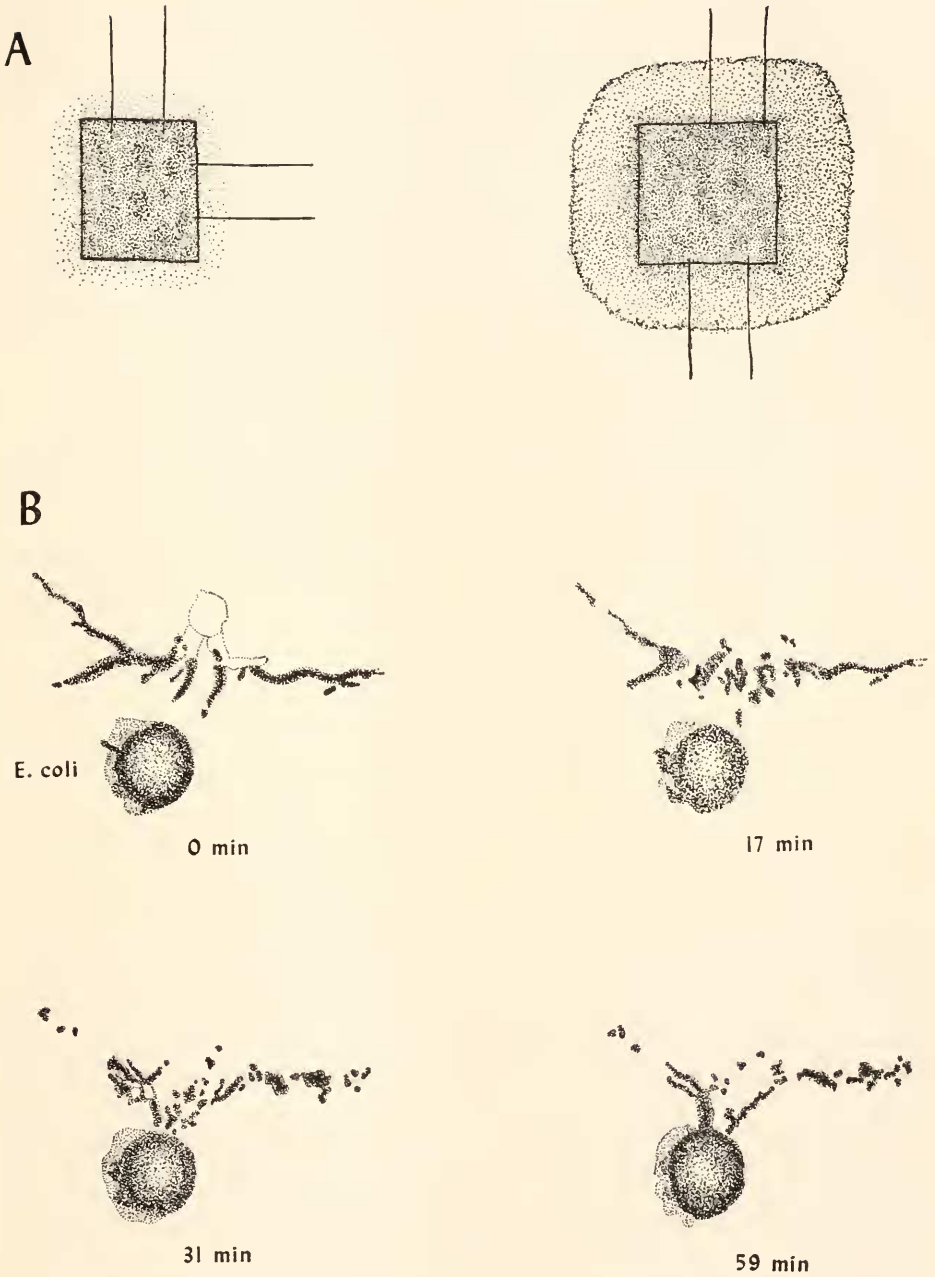

FIGURE 1. A. Drawings of two photographs of the assay. The control is at the left, and an experimental with active rate substance is shown on the right. The central cellophane square is about 4 mm. long. B. Drawings of a series of photographs showing the effect of a colony of *E. coli* on aggregating amoebae. In the first illustration (0 min.) the outline of the position of the original center before removal is indicated. The width of the *E. coli* colony is about 300 μ.

TABLE I

Four experiments and their controls picked at random. The first two are measured over a 4-hour period and the second two over a 2-hour period. Each column of 6 figures represents the 6 marked sides of the 3 cellophane squares on one petri dish. Each figure is the mean of the 4 fastest amoebae at about 1 hour subtracted from the 4 fastest at 2 (or 4) hours later. Since the controls vary somewhat (possibly due to moisture conditions) the experimental figure can be considered in terms of per cent increase over the control. The standard deviations are of the 6 means (of 4 amoebae each). This is equivalent to the standard error of the average of all the 24 amoebae involved in each test.

Date	Control in mm./hr.	Mean	Standard deviation	Experimental in mm./hr.	Mean	Standard deviation	% Increase of experimental
9 April '65	0.33			0.71			
	0.37			0.79			
	0.34			0.71			
	0.30			0.66			
	0.36			0.71			
	0.31	0.34	0.03	0.70	0.71	0.04	109%
13 April '65	0.35			0.78			
	0.31			0.70			
	0.42			0.87			
	0.36			0.86			
	0.30			0.72			
	0.42	0.36	0.05	0.76	0.78	0.07	117%
26 May '65	0.30			0.92			
	0.32			0.95			
	0.23			0.94			
	0.25			0.73			
	0.21			0.90			
	0.23	0.26	0.04	0.90	0.89	0.08	242%
31 May '65	0.35			0.68			
	0.29			0.58			
	0.30			0.68			
	0.37			0.63			
	0.41			0.49			
	0.38	0.35	0.05	0.56	0.60	0.08	71%

few the only disadvantage was that it was difficult to find enough cells to measure, but should they be found, their rate of outward movement was the same as with dense populations, all other conditions being equal. In general we found that fairly concentrated amoeba densities were the most convenient. We attempted roughly to cover the cellophane with a solid layer of cells, but we knew that any variation did not affect the test results.

Finally some experiments were run to see if any of the more obvious solutes in the test agar might affect the rate of movement. In agreement with the results of Samuel (1961), phosphate buffer, standard salt solution, as well as non-toxic concentrations of NaCl had no effect on the rate of movement. Also 0.1% peptone was tried in the test agar, again with negative results.

Quantitative nature of the assay

The first indication that the test was quantitative came with some early experiments with single amoebae. If the concentration of the test solution was diluted,

the speed was apparently reduced. To determine the exact relation a series of 1/5 dilutions over a 25-fold range were run using the cellophane square assay method and in 9 separate experiments it was shown that a 5-fold increase in concentration produces a 1.2-fold increase in the rate of movement. It is not known at present over how great a range of concentration this relation holds, although when the chemical work is at a more advanced stage this question will be pursued further. Under ideal conditions the plain agar controls run about 0.30 mm./hr., and with very active solutions it is possible to obtain speeds over 1.00 mm./hr. If the rate/concentration ratio holds over this range, then if we arbitrarily give a rate of 0.39 mm./hr. a concentration of 1, then 1.00 mm./hr. should indicate a concentration between 10^3 and 10^4 times greater.

Shaffer tests

Throughout the experiments to be described periodic Shaffer tests were performed, following the procedure outlined by Shaffer (1956b). For any one test solution the tests were run numerous times to make certain of the result. Even a positive result must be interpreted with caution for it is always possible that new centers arise near the edge which could attract the amoebae. To give an idea of the type of results obtained, one solution was tested in 19 separate Shaffer tests. In 8 tests the result was clearly positive; in 11 tests the result was doubtful or clearly negative where there was no hint of orientation towards the edge of the agar block. From this experiment it was concluded that the test solution did contain an orienting, chemotactic factor. The variation in the results is presumably due to differences in the conditions of the amoebae (which must be in the process of aggregating before they are placed under the block) and to the skill of the operator in the handling of the agar blocks.

Results

Methods of obtaining active solutions

The original solutions which were used to perfect the test were prepared by a method devised by Francis (1965), based upon the original technique of Shaffer (1956a, 1956b). It consists of washing cells by centrifugation and allowing them to settle on the bottom of a glass dish containing distilled water. As soon as aggregation begins the water is changed for fresh distilled water, which, after 30 minutes over the aggregating amoebae, is removed and as quickly as possible brought to a boil. In these early experiments and the majority of all subsequent experiments we concentrated this original solution 200 times by boiling. This was usually done by bringing the preparation to dryness and then redissolving the residue in enough water to give a 200 × concentration.

Once the test was devised, immediately attempts were made to improve the method of obtaining more active solutions. We will not discuss here (for the work is still in progress) the chemical methods of purification and concentration, but rather the basic method of obtaining active solutions from aggregating amoebae. But before we proceed any further it may be wise to give a provisional name to the active substance. We cannot call it acrasin since that substance is defined as being involved in aggregation chemotaxis and here we are testing for rate of movement.

Even though the preparations have been tested at intervals by the Shaffer test with positive results, we cannot be sure as yet that there are not two or more substances. It is even conceivable that there is more than one orienting substance but all of these matters must await the results of the chemical analysis before they can be settled. Since here we are testing primarily for the substance which increases the rate of movement of the amoebae let us for the time being call it the *rate substance,* and leave in abeyance the question of whether or not this is the same substance as acrasin.

In this series of experiments the amoebae that were used to produce the rate substance were prepared by the liquid nutrient medium devised by Sussman (1961). The amoebae were fed with *E. coli* B/r and shaken for 48 hours at 22° C. In order to reduce the labor, the amoebae were sometimes suspended in Pyrex baking dishes (35 × 22 cm.) in distilled water about a centimeter deep, although these gave less consistently satisfactory results than the petri dishes, presumably because the amoeba density was more difficult to control. (The reason for not giving exact data on amoeba density will soon be clear; better methods of obtaining the rate substance have been devised.)

One of the difficulties was that there often was a considerable precipitate after boiling. To circumvent this the amoebae were placed in dialyzing bags with distilled water and these were placed in a Pyrex baking dish filled with distilled water. If these were set up in the evening, by next morning it could be seen that aggregation had occurred within the bags. The water outside the bags was poured off into a beaker and boiled down 200×. It gave a clear and precipitate-free solution, and although it showed considerable rate substance activity, it had roughly the same potency as the solution prepared without the dialysis bags and concentrated 200×. The controls gave an average of 0.37 mm./hr. (four cases); two cases in which the bags were gently shaking averaged 0.48 mm./hr., and two cases in which the dishes were still averaged 0.55 mm./hr. Note that there is a slight advantage when the preparation is not agitated.

As there was no appreciable gain from the method involving dialysis bags, the simpler method involving the decanting of the water from over the amoebae was resumed. It was found that the precipitate could be effectively removed by filtering and we were able to show that one could filter either after the solution was first brought to a boil or after it had been boiled down; the activity was about the same for both methods.

Two experiments were performed to test the possibility that some of the potency of the solutions was lost in boiling. Instead of boiling the solution it was concentrated 40× in a flash evaporator which takes off the water in a vacuum at 40° C. In two experiments the controls averaged 0.35 mm./hr., the vacuum evaporated 0.34 mm./hr., and a further aliquot reduced 40× by boiling gave a mean value of 0.48 mm./hr.

It is clear from this experiment and all the others reported in this section that the original boiling method of isolation is the simplest and most effective. The fact that all the activity was lost during the flash evaporation at 40° C. suggests that, similar to the observations of Shaffer (1956a, 1956b) and Sussman, Lee and Kerr (1956), an enzyme has been retained which specifically destroys the rate substance. Unfortunately whether or not this is the correct interpretation is still

in doubt despite a number of experiments that have been run to clarify this point. Again it is hoped that when the chemical nature of the rate substance is known this matter will be settled.

Species specificity

A series of experiments were run involving *D. discoideum* (Strain No. 1), *D. mucoroides* (Strain No. 11) and *Polysphondylium violaceum* (Strain No. 6). Using our rate test it was impossible to show any species specificity. The rate substance prepared from *D. mucoroides* stimulated the amoebae of *D. discoideum* and *vice versa;* the rate substance of one was indistinguishable from the other in its effects. The only discernible difference was that *D. mucoroides* provided poor test amoebae (as did *P. violaceum*) in that the amoebae tended to aggregate too quickly and, especially in the controls, few amoebae escaped from the cellophane squares (Table II).

The same result was observed between *D. discoideum* and *P. violaceum*. *P. violaceum* rate substance was tried numerous times on *D. discoideum* test amoebae and again its effect equalled that of the *D. discoideum* rate substance.

This lack of genus specificity was surprising, in the light of the observations of Shaffer (1957a) who has presented good evidence that there is a separate *Polysphondylium* and *Dictyostelium* acrasin, although the former species produces both. However, Shaffer does point out that it is possible to interpret his result on the basis of one substance if one makes certain assumptions concerning a differential response to it by the two species.

To examine this question further we ran 10 Shaffer tests with our 200× concentration rate substance preparations of *P. violaceum* on *D. discoideum* test

TABLE II

The effect of the rate substance of different species upon one another (figures expressed in mm./hr.)

	D. discoideum test amoebae		*P. violaceum* test amoebae	
	Controls	Experimentals	Controls	Experimentals
D. discoideum rate substance	0.37	0.61 0.55	0.16	0.79 0.73
D. mucoroides rate substance	0.32	0.40		
P. violaceum rate substance	0.30	0.48	0 (aggregated)	0.45
	0.26	0.48	—	0.40
	—	0.50		
	—	0.49		
	—	0.45		
	—	0.40		
	—	0.51		
	0.34	0.40		

amoebae. In 6 of the tests the result was positive and there was strong orientation towards the meniscus, while the remaining four cases were doubtful or negative. There is, therefore, good evidence that a concentrate of water which has been sitting over *Polysphondylium* amoebae contains both a rate-increasing and an orientation factor for *Dictyostelium* amoebae. However, this completely evades the question of species specificity which clearly exists; it is a matter which will require much further work.

Effect of the rate substance preparations on the appearance of the amoebae

During the course of the assays a number of striking features in the appearance of the amoebae on the dishes containing the rate substance preparations were observed that were absent in the controls. It must again be cautioned, however, that we cannot at this time be certain that these effects are produced by the rate substance itself, but it is possible that they may be produced by some associated substance.

If the preparation is effective, and the rate higher than about 0.70 mm./hr., then there is often a sharp advancing edge in the experimentals that is totally absent in the controls (Fig. 1A). This resembles the feeding edge observed by numerous workers although here, of course, there are no bacteria and the cells are not feeding.

If, in a similar test with an active rate substance preparation, the test amoebae are about to aggregate, then another property is sometimes evident. The cells at the outer edge tend to adhere to one another in groups. Even more remarkable, on rare occasions the cells actually form streams which lead outward to the advancing edge. These streams never form centers and eventually they dissolve. Again, neither the streams nor the adhesive properties of the cells are seen in the controls.

It is of interest to note here that a similar pattern of streams without centers radiating away from a central point may be seen in the disintegration of the primary centers, and the formation of secondary centers in *D. minutum* as described by Gerisch (1964).

Effect of rate substance preparation on center formation.

The process of center formation and the inhibition or stimulation of centers is one that recently has received considerable attention (Bonner and Dodd, 1962; Bonner and Hoffman, 1963; Shaffer, 1963; Hohl and Raper, 1964; Gerisch, 1964; Kahn, 1964). Since centers will form directly upon the cellophane squares, and since the squares for the controls and the experimentals are prepared in the same way, it is possible to test whether or not the rate substance preparations will affect the number of centers per unit area (or the aggregation territory size).

It is very obvious that the test preparations do not affect the number of centers on the cellophane squares; the experimentals always resemble the control regardless of how effective the preparation is in increasing the rate of movement of the amoebae.

When is the rate substance produced and what kind of cells produce it?

These experiments began with asking the question of what periods, before, during and after aggregation, were the most active in producing the rate substance.

The amoebae after 48 hours incubation were washed by centrifugation and placed on the bottom of the Pyrex baking dishes under a layer of distilled water. This was done at 5 PM and the first collection of water to be concentrated was taken after three hours. During this time no aggregation had occurred. Fresh distilled water was added immediately over the amoebae and they were allowed to stand overnight (13 hours). This water was removed and concentrated, again adding fresh water to the dishes. One or two three-hour samples were collected the same way in the morning and each one was concentrated 200 ×. During this period aggregation had been completed and small centers were evident.

When these samples were tested the controls gave an average of 0.29 mm./hr. (four cases); the first three hours an average of 0.74 mm./hr. (four cases); the next 13 hours, 0.51 mm./hr. (three cases); the next three hours, 0.53 mm./hr. (two cases); and the final three hours, 0.53 mm./hr. (one case). It was not surprising to find that the stages after aggregation showed activity for it is well known that these stages produce acrasin, but it was most unexpected that the rate substance should be produced long before aggregation begins. The fact that the first reading was higher than the others is possibly not significant for it might contain more cells. When the cells are placed in distilled water they do not adhere well to the glass bottom and as a result many are lost with the first decanting.

To control this factor a series of five baking dishes were set out, care being taken that they contained exactly the same number of amoebae as well as the same amount of water. These were allowed to remain different periods of time, and each was poured off and boiled down exactly 200 ×. The entire experiment was run twice. The results of each were consistent and therefore they have been averaged. The controls gave 0.35 mm./hr. The 5-minute sample was 0.79 mm./hr.; the 15-minute sample 0.70 mm./hr., the 30-minute sample 0.69 mm./hr., the 120-minute sample 0.67 mm./hr., and the overnight (19-hour) sample 0.52 mm./hr.

The surprising result here is that in 5 minutes, again long before aggregation, more of the rate substance is given off into the water than any of the longer periods. There is, in fact, a clear decline in activity if the water is left standing over the amoebae for 19 hours.

The next step was to test the supernatant right in the testtube, directly following the second centrifugation of the amoeba-washing process. This was compared to the whole contents of the testtube (cells and supernatant) as well as the cells alone. It should be noted here that the absolute values have little meaning, for the amount of concentration is much less here since the original volume of water is less. Controls averaged 0.40 mm./hr. (three cases); the cell-free supernatant averaged 0.55 mm./hr. (two cases); the cells and the supernatant combined gave a value of 0.75 mm./hr. (two cases); and the cells alone were 0.68 mm./hr. (two cases). In one experiment the cells were sonicated and the resulting cell-free preparation gave a value of 0.76 mm./hr. It is clear from this that the cells themselves, long before aggregation, not only are continually giving off the rate substance, but the substance is contained within the cells also.

As an additional control, a suspension of *E. coli* was prepared, boiled down, and tested with the astonishing result of very high activity. Not only *E. coli*, strain B/r, but other strains which had been stored dry or frozen. In 9 experi-

ments, lumping all the different kinds of *E. coli* preparations, the control values were 0.35 mm./hr. while the *E. coli* values were 0.94 mm./hr. These gave greater activity than had hitherto been observed. In many of the tests the exact concentration was unknown although a 5% preparation made with dry *E. coli* was especially effective. The highest value obtained was a rate of movement of 1.22 mm./hr., when the control ran 0.23 mm./hr. This is a 430% increase in the velocity of the cells.

The substance given off by the *E. coli* is also dialyzable and in fact has all the properties of the amoeba rate substance. The morphology of the cells under the influence of the rate substance derived from *E. coli* is indistinguishable from that of the amoebae. The *E. coli* rate substance was tested with the Shaffer test and in 19 tests, 8 were positive and 11 doubtful or negative, indicating without question that the *E. coli* rate substance possessed the ability to orient aggregating amoebae.

The final and most elegant demonstration of this fact can be readily made with a simple and direct experiment. Amoebae of *D. discoideum* were grown on non-nutrient agar, over which had been spread a thin layer of *E. coli,* the standard method of preparing the cells for the Shaffer test. When aggregation was in full progress, the center of an aggregate was removed by a hair loop and a small drop of fresh *E. coli* cells was placed about 100 μ from the side of a large stream. Within 15 minutes, the cells of the stream will break up and by 30 minutes they will form chains of cells leading into the colony of *E. coli* as though it were a center (Fig. 1). If the removed center is placed in a similar spot for comparison, it is clear that the normal slime mold center is stronger in orienting the aggregating cells and the cells respond more rapidly. But the fact that the streams of aggregating amoebae will form into colonies of *E. coli* is absolutely clear-cut.

Some experiments were run with yeast extract and it was found that this also contained a rate-increasing factor although in general it was less satisfactory as a source for the rate substance in that it apparently contained substances that were toxic to the amoebae as well. In three experiments the average of the controls was 0.35 mm./hr. while 1% yeast extract gave an average value of 0.65 mm./hr. It should be pointed out that since we can now obtain our rate substance from bacteria and yeast, the chemical analysis will be greatly facilitated; quantity is no longer a problem.

Evidence that the repulsion between cells does not involve the rate substance

As has already been discussed, if different concentrations of amoebae are placed on the cellophane square in the assay, there is no effect on the rate of movement of the cells. This is the first indication that the substance which is responsible for the repulsion between the amoebae is distinct from the rate substance. To test this possibility further, small blocks of agar containing a high concentration of the rate substance (*ca.* 0.70 mm./hr.) were placed different distances from an advancing edge of amoebae coming off a cellophane square in a control test petri dish. In no case was the advancing edge stopped by the presence of the rate substance in the block. In fact, quite to the contrary, the cells on that side of the cellophane square were stimulated in their rate of movement and passed right over the block, spreading away from the cellophane square at an

increased pace. The evidence supports the notion that the mechanism of the repulsion between cells is totally independent of the rate substance.

Discussion

We have at the moment five recognizable phenomena that have the appearance of being related. This appearance, however, could be quite misleading and we cannot know their relation until the substances involved have been isolated and identified.

The five phenomena are: (1) the orientation of amoebae in normal aggregation; (2) the orientation of amoebae to test substances in the Shaffer test; (3) the orientation of the vegetative or pre-aggregation amoebae to colonies of *E. coli;* (4) the orientation of aggregating amoebae towards colonies of *E. coli;* (5) the increase in rate of the amoebae at various stages. There are undoubtedly other phenomena as well, involving both rate and orientation of which we are still ignorant.

One extreme in the interpretation of the results accumulated thus far is that each of these phenomena is mediated by a separate substance; in fact they could conceivably each involve more than one.

The other extreme, and one which obviously has more appeal, is that they are all mediated by one substance. As has been said before, this matter is currently under investigation (with the generous help and advice of Dr. A. B. Pardee and his associates), but it may still be some while before it is resolved. In the interim let us see what are the present arguments to favor such an hypothesis and, if it is assumed to be the case, what are the consequences as far as slime mold development is concerned.

The preparations of rate substance have the property of inducing adhesiveness and stream formation, a property characteristic of acrasin, as Shaffer (1957b) showed. This has the flaw that many substances might have this property, or that the acrasin has not been separated from the rate substance.

The idea that rate increase and orientation might go hand in hand is consistent with one old observation. It has long been known from time-lapse films of aggregation that the cells often move inward in waves or pulses that start in the center and radiate outward. It has been assumed that these are pulses of acrasin secretion, and the pertinent point is that the outgoing waves are characterized by periods of fast inward movement of amoebae. In other words if the pulses are indeed periodic puffs of acrasin, these puffs simultaneously accelerate and orient the amoebae.

Another observation made from an old film would also fit in with the idea that rate and orientation go together. When a center is removed during aggregation the streams break up, but they continue to pulse; the cells repel one another but do so in definite intervals of fast movement. If, as it has always been assumed, the pulses are associated with acrasin secretion, then acrasin continues to be emitted in puffs, yet there is no centripetal acrasin orientation.

This paradox is eliminated if we consider aggregation to be characterized by a period of especially high orientation sensitivity to acrasin, so high in fact that it overrules the separate cell repulsion phenomenon. According to this hypothesis, then, the removal of the center caused a momentary loss of orientation sensitivity

on the part of the cells, not a loss in acrasin secretion, the existence of which is demonstrated by the pulses of fast movement.

This would also explain why the Shaffer test only works with amoebae that are in the process of aggregation, and not cells just about to aggregate. One must have cells which are in this state of high sensitivity.

The important point is that if we assume all orientation and rate-increasing to be achieved by one substance, then our traditional picture of the mechanism of aggregation must be radically altered. We can no longer think of the onset of aggregation as the moment when cells begin both to secrete and become sensitive to acrasin. Acrasin may be present very early, even in the food source, and is presumably a means of the amoebae reaching the food. There is a weak ability to orient the amoebae at this stage, as Samuel (1961) showed, but the separate cell repulsion phenomenon keeps the cells to some degree dispersed. After the food supply is depleted something occurs rather suddenly within the amoebae that produces a great increase in orientation sensitivity. As a result a single chemotactic factor can be used in the first instance as a means of finding food, and in the second as a means of aggregating and forming a communal spore-bearing structure.

Along with cell repulsion, it is clear that center formation and center inhibition are distinct processes from the orientation-rate factors described above. Besides these separate problems there are a number of others that need thorough investigation. For instance, there is the whole matter of how gradients of acrasin arise. This may be related to the interesting observations of Samuel (1961) who showed that the amoebae had different rates during different periods of their development, the period just prior to aggregation being one of especially slow movement. Another matter that needs further investigation is that of species specificity. Furthermore, the whole question of the repulsions (Bonner and Dodd, 1962) and attractions (Shaffer, 1964) of pseudoplasmodia in the air is puzzling and there is no information as to whether or not it relates in any way to the rate substance described here. Another point that should receive attention is that of the rate movement of the slugs. There have been conflicting opinions as to why larger slugs move faster than smaller ones (Bonner and Eldredge, 1945; Bonner, Koontz and Paton, 1953; Francis, 1962; Shaffer, 1962, 1964, 1965), and now we can suggest the further hypothesis that the larger slugs simply contain more of the rate substance.

Even if it turns out that the rate substance is utterly distinct from the orienting acrasin, its significance will be considerable, not only as to its role in slime mold development, but its role in the physiology of amoeboid movement. There are many new avenues of investigation that have been opened up by this approach.

Summary

1. It has been shown that there is a substance (or substances) given off by the cellular slime mold amoebae that both orients aggregating cells (*e.g.* in the Shaffer test) and increases the rate of movement of the cells. A simple, quantitative assay has been developed to test the rate of cell movement. Using both types of test in conjunction, we have been able to show that the substance(s) is produced

before the aggregation of the amoebae and is even present in large amounts in the food source, *E. coli*.

2. The substance(s) is not species-specific; it induces adhesiveness in cells approaching aggregation; it does not affect center formation or center inhibition (territory size); nor is it related to the phenomenon of cell repulsion.

3. It is quite possible that there are many substances involved and the rate-increasing substance(s) is separate from orientation substance(s). Another possibility is that they are all one substance, which has hitherto been called acrasin. If this is the case then aggregation can no longer be considered the moment of first acrasin secretion, but one would have to assume that it is the moment of first acute sensitivity to acrasin, a sensitivity sufficient to override the cell repulsion phenomenon. It is hoped that if the chemical identification of the rate substance is achieved, then many of these important questions will be answered.

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